Supplementary Materials and Methods

Endothelial cells culture

BOECs were isolated as previously described with minor modifications[22]. Cells were pooled from 3-4 donors and cultured in EGM-2 containing 20% Fetal Bovine Serum (FBS). For SILAC labeling, BOECs were cultured in SILAC EBM-2 (w/o arginine and lysine, custom made, Lonza), lysine 0 and arginine 0 (light 'L') (Sigma) or lysine 4 and arginine 6 (medium 'M') or lysine 8 and arginine 10 (heavy 'H') (Cambridge Isotopes), supplemented with bullet kit and 10% FBS (passage 2-4) or 1kDa in house dialyzed FBS (passage 5-7). At P7, cells were fully SILAC labeled and used for experiments. Prior to the experiment, BOECs were washed twice with Hank's Balanced Salt Solution (HBSS, Invitrogen) and starved for 2 h in SILAC EBM-2. Cells were stimulated with 1U/ml high-activity thrombin (1500-3500 National Institutes of Health (NIH) units/mg protein, T4393, Sigma) for 2, 5, 10 or 30 min (3×15 cm culture dishes per condition). Cells were washed once with HBSS and lysed with SDS lysis buffer, 4% SDS, 100 mM ditiotreitol (DTT), 100 mM Tris pH 7.4, phosphatase and protease inhibitor cocktail (Thermo Scientific). Cell lysates were incubated 5 min at 95°C, sonicated, centrifuged 10 min at 16,000g and the cleared lysates snap-frozen. Heavy, light and medium supernatants were pooled, centrifuged for 5 min at 200g and snap-frozen. Experiments were performed in triplicate, swapping the three different labeling conditions.

Immunofluorescence

SILAC labeled BOECs were grown to confluence on gelatin-coated glass coverslips, stimulated with thrombin as described above and fixed with ice-cold 3.7% paraformaldehyde in PBS. Immunofluorescence analysis was performed as previously described[24]. Monoclonal CLB-Rag20 (IgG_{2b})[25] and CLB-HEC75 (IgG_1)[26] were used to stain von Willebrand factor and Platelet/Endothelial Cell Adhesion Molecule 1, respectively. Alexa Fluor 488 goat anti-mouse IgG_{2b} and Alexa Fluor 568 goat anti-mouse IgG_1-conjugated secondary antibodies were from Invitrogen. Cells were embedded in Mowiol® 4-88 (Polysciences, Inc.) and viewed by Confocal Laser Scanning Microscopy with a 63x/1.4 oil objective (LSM510, Carl Zeiss).

Western blots

Cells were stimulated with thrombin as described above and lysed with 2xSDS-PAGE buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue) supplemented with 100 mM DTT and phosphatase and protease inhibitor cocktail (Thermo Scientific). Cell lysates were incubated 5 min at 95°C, passed 20 times through a 23G needle (BD Biosciences), separated on a 4%-12% NuPAGE Novex Bis-Tris gel (Life Technologies) and blotted on a PVDF membrane (Life Technologies) (rabbit anti-pMyosin Light Chain 2 (Thr18,Ser19) (3674, Cell Signaling) or nitrocellulose membrane (Life Technologies) (mouse anti-pERK (E-4) (Tyr204) (sc7383, Santa Cruz) and rabbit anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (9211, Cell Signaling)). Mouse anti-α-tubulin (DM1A clone, Sigma) was used as a loading control and horse-reddish peroxidase labelled sheep anti-mouse (NA931V, GE Healtcare) and donkey anti-rabbit (NA934V, GE Healtcare) were used as secondary antibodies. Western blots were developed using BM chemiluminescence blotting substrate (POD) (Roche).

Phosphoproteome

Global phosphoproteome. Equal amount of light, medium and heavy SILAC-labeled lysate were mixed together and processed as previously described[16,17]. Briefly, mixed proteins were reduced, alkylated and digested with trypsin using the FASP method[27]. Digested peptides were acidified to pH 2.5 with TFA and separated with strong cation exchange (SCX) chromatography (Resource S column, GE Healthcare) using 5 mM KH_2PO_4 pH 2.7, 30% acetonitrile (ACN) and 5 mM KH_2PO_4 pH 2.7, 30% ACN, 350 mM KCl buffers. SCX fractions were pooled into 6 according to UV absorbance and phosphopeptides enriched using TiO_2 beads (GL sciences) in the presence of 2,5-dihydroxybenzoic acid (DHB)[28]. Phosphorylated peptides were eluted with 15% ammonium hydroxide, 40% ACN, loaded onto Empore-C_{18} StageTips[29], eluted with 80% ACN, 0.5% acetic acid, and stored at -80°C until MS analysis. Also the SCX flow through was collected and processed as described above performing 4 consecutive rounds of TiO_2 enrichment, which were run separately at the MS.

Secretome

The light, medium, heavy supernatants were combined and ultra-centrifuged at 4°C for 70 min at 100,000g. Proteins were isolated using silica-based resin (J.R.H., M.B. and S.Z., manuscript in preparation), dissolved in 4x sample buffer (NuPAGE LDS loading buffer, Life Technologies) supplemented with 0.1 M DTT, incubated for 5 min at 95°C and separated on a 4%-12% NuPAGE Novex Bis-Tris gel (Life Technologies). Each gel lane was cut in seven slices and proteins in-gel digested with trypsin[30]. Peptides were loaded onto Empore-C_{18} StageTips[29], eluted with 80% ACN, 0.5% acetic acid and stored at -80°C until MS analysis.

Mass spectrometry analysis

Digested peptides were separated by nanoscale C_{18} reverse chromatography (Easy nLC, Thermo Scientific) coupled on line to a linear trap quadrupole (LTQ)-Orbitrap Elite mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on 20 cm fused silica emitter (New Objective) packed in-house with ReproSil-Pur C_{18}-AQ, 1.9 μm resin (Dr Maisch GmbH) and eluted with 0-30% solvent (80% ACN, 0.5% acetic acid) over 90 min. Full scan MS spectra were acquired in the Orbitrap analyzer with a resolution of 120,000 at m/z 400, and a target value of 1,000,000 charges. The 10 most intense ions were selected for high collision dissociation fragmentation with a target value of 40,000 charges and acquired in the Orbitrap with resolution of 15,000 at m/z 400 Th. All data were acquired with Xcalibur software.

MS data analysis

The RAW MS files were processed with the MaxQuant computational platform, 1.3.6.0. Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (release-2012 01, 81,213 entries)[S1]. To search for precursor and fragment ions, an initial maximal mass deviation of 7 ppm and 20 ppm, respectively, was required. Trypsin with full enzyme specificity and only peptides with a minimum length of 7 amino acids were selected. A maximum of two missed cleavages was allowed. Carbamidomethylation (Cys) was set as fixed modification, while Oxidation (Met) and N-acetylation as variable modifications, as well as phospho(STY) for the phosphoproteome

analysis. For protein, peptide and phosphorylation site identification, we required a maximum false discovery rate (FDR) of 1%. The relative quantification based on SILAC was performed by MaxQuant enabling the ‘re-quantify’ and ‘match between runs’ options.

Data analysis and normalization

Data processing and annotation were performed using the Perseus module of MaxQuant version 1.3.6.0 (for most of the analysis) and 1.4.1.10 (for the functional annotation of the sites). First, we eliminated from the MaxQuant output files the reverse and contaminant hits. For the secretome, only proteins identified with at least 1 unique peptide and quantified with a minimum of two ratio counts were considered for the analysis. For the phosphoproteome, only class I phosphorylation sites, which refer to phosphorylation sites where the phosphorylation could be assigned with a localization probability of at least 0.75 and a score difference ≥ 5[16], were used for the analysis. For each experiment, the normalized SILAC ratios were transformed using the binary logarithm (log_2). For each phosphorylation site, the SILAC ratios were calculated separately for singly, doubly or multiply phosphorylated peptides. Annotations were added based on the Uniprot IDs of each entry (GO[32] and KEGG[S2] categories) or based on the Uniprot IDs and sequence window (Kinase motifs based on Hprd database[S3]; known sites and known/predicted kinases based on UniProt[S1] and PhosphoSitePlus[S4] databases; functional sites based on PhosphoSitePlus database).

Category enrichment and kinase analysis

The regulated phosphorylation sites (2224) were divided into the minimum number of allowed clusters, six (Figure 2A), using the hierarchical clustering pre-processed with K-mean option available in Perseus. Category and motif enrichment analysis was performed using a Fisher exact test comparing the annotation of the phosphorylated proteins or phosphorylation sites belonging to each cluster to the complete dataset. For the secretome, the subset of proteins with increased secretion was compared to the complete secretome dataset. Multiple hypothesis testing was controlled by using Benjamini-Hochberg FDR. For the GO enrichment analysis, a FDR of 7.5% (phosphoproteome Figure 2B) and 2% (secretome Figure 7D) and a minimum enrichment factor higher than 1 were

required. For the motif analysis (Figure 2D), a FDR of 5%, a minimum enrichment factor higher than 1 and a minimum number of occurrences of 5 were required. The enrichment factor was calculated as (Intersection size/Category size)/(Selection size/Total size) (see Supplementary Tables S3, S5). We determined the significantly overrepresented linear kinase motifs with Motif-X[33] by querying, for each cluster, the sequence motifs of the phosphorylation sites against the IPI human database, using a p-value of E-6 and a minimum number of occurrences of 5.

Supplementary Tables and Figures

Table S1. Identified class I phosphorylation sites in thrombin-stimulated BOECs.

Serine, threonine, and tyrosine phosphorylation sites identified in the six experiments. A1,A2,A3 indicate 3 reversed labeling experiments of time points 0, 2 and 10 minutes and B1,B2,B3 indicate 3 reversed labeling experiments of time points 0, 5 and 30 minutes. _1, _2, and _3 indicate that the quantification was based on singly, doubly, or multiply phosphorylated peptides, respectively. NaN = not a number (not quantified).

Table S2. Class I phosphorylation sites quantified in at least 2/3 experiments in each of the time points.

This table contains the normalized SILAC ratio of the phosphorylation sites quantified in at least 2/3 experiments in each of the time points in thrombin-stimulated BOECs. The information contained in this table has been used for the bioinformatic analysis. The column Multiplicity indicates if the quantification of the phosphorylation site was based on singly (_1), doubly (_2), or multiply (_3) phosphorylated peptides. Up and down regulated phosphorylation sites belonging to the six temporal clusters are indicated. NaN = not a number (not quantified).

Table S3. GO and motif analysis of the phosphoproteome.

List of GO biological process, cellular component and molecular functions categories ("GO enrichment sheet") and motifs ("Motif enrichment" sheet) enriched in the phosphoproteome following a Fisher exact test (for details see Supplementary Materials and Methods).

Table S4. Identified protein groups in the secretome.

Protein groups identified in the six secretome experiments. A1,A2,A3 indicate 3 reversed labeling experiments of time points 0, 2 and 10 minutes and B1,B2,B3 indicate 3 reversed labeling experiments of time points 0, 5 and 30 minutes. Early and late secreted protein groups are indicated. NaN = not a number (not quantified).

Table S5. GO and motif analysis of the secretome.

List of GO biological process, cellular component, molecular function and KEGG categories enriched in the secretome following a Fisher exact test (for details see Materials and Methods).

Table S6. Thrombin-regulated phosphosites belonging to proteins containing "transcription factor activity" within the GOMF annotation text.

Figure S1. BOECs phosphoproteomics.

(A) Phosphoproteomic workflow. H = Arg10Lys8 (heavy); L = Arg0Lys0 (light); M = Arg6Lys4 (medium).

(B) Light microscopy of SILAC-BOECs.

(C) Immunostaining for PECAM1 (green) and von Willebrand Factor (red) of SILAC-BOECs. Bar = 50 µm. LSM510 confocal laser scanning microscopy (Carl Zeiss), 63x/1.4 oil objective.

(D) Zoom of the boxed area in panels (C). Bar = 50 µm.

(E) Mean and standard deviation of Pearson correlation coefficients of the phosphoproteomes, calculated between replicates and time-points.

(F) Histogram of the all SILAC ratio ($\log_2$) for the quantified phosphorylation sites reported in Table S2. Highlighted are the thresholds (≥ one standard deviation) used to define up and down regulated phosphosites. Counts = number of phosphosites.

Figure S2. Distribution normalized SILAC ratios.

Histogram distribution (x axis between -0.75 and 0.75) of the normalized SILAC ratios calculated for the identified protein groups (based on non-phosphorylated peptides identified by MS). A1,A2,A3 indicate 3 reversed labeling experiments of time points 0, 2 and 10 minutes and B1,B2,B3 indicate 3 reversed labeling experiments of time points 0, 5 and 30 minutes.

Supplemental references

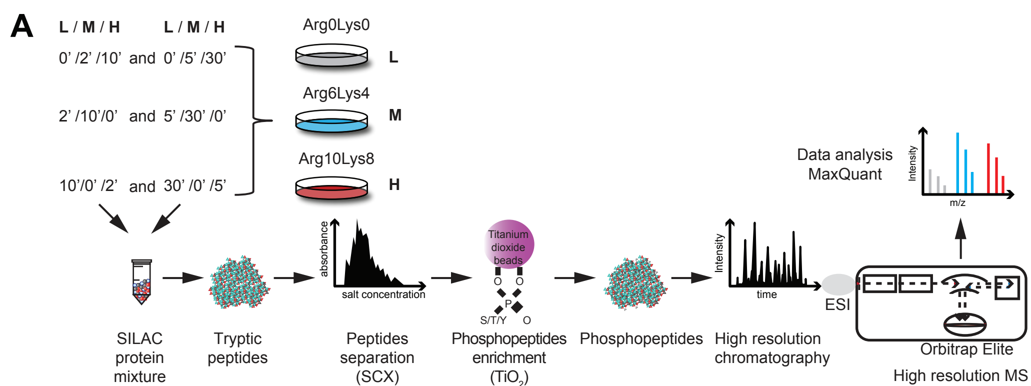
S1. UniProt C. The Universal Protein Resource (UniProt) in 2010. *Nucleic Acids Res*. 2010;38(Database issue):D142-148.

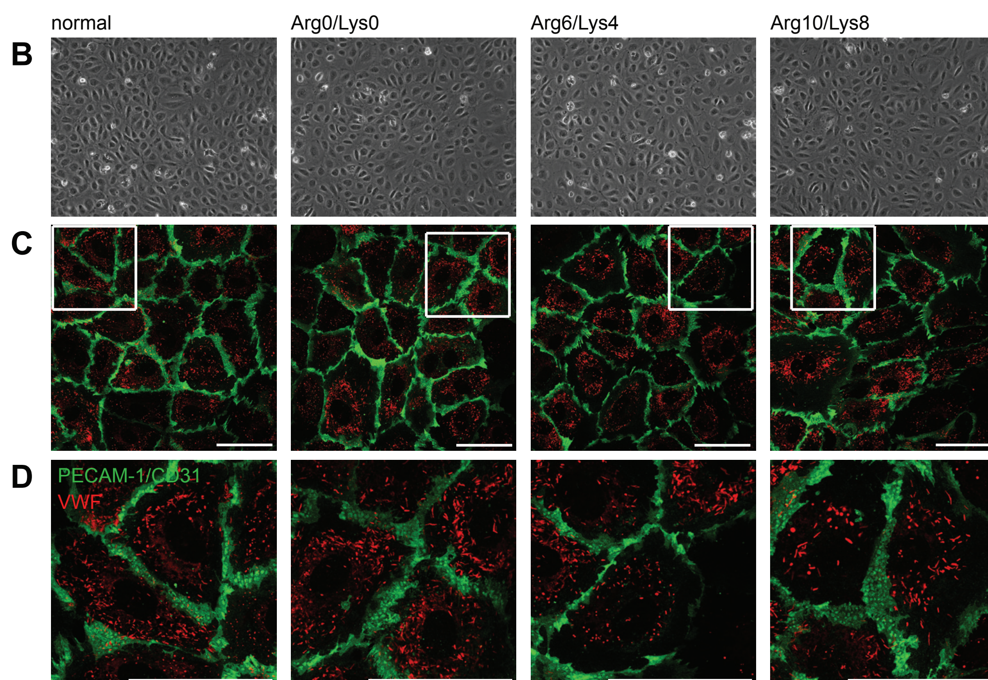
S2. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res*. 2012;40(Database issue):D109-114.

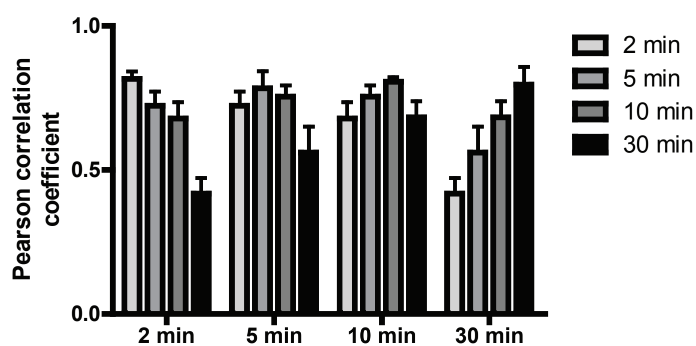
S3. Keshava Prasad TS, Goel R, Kandasamy K, et al. Human Protein Reference Database--2009 update. *Nucleic Acids Res*. 2009;37(Database issue):D767-772.

S4. Hornbeck PV, Kornhauser JM, Tkachev S, et al. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res*. 2012;40(Database issue):D261-270.

Figure S1







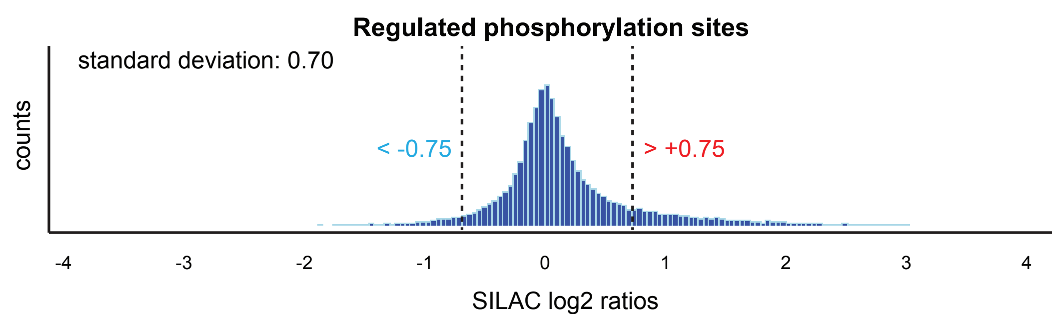
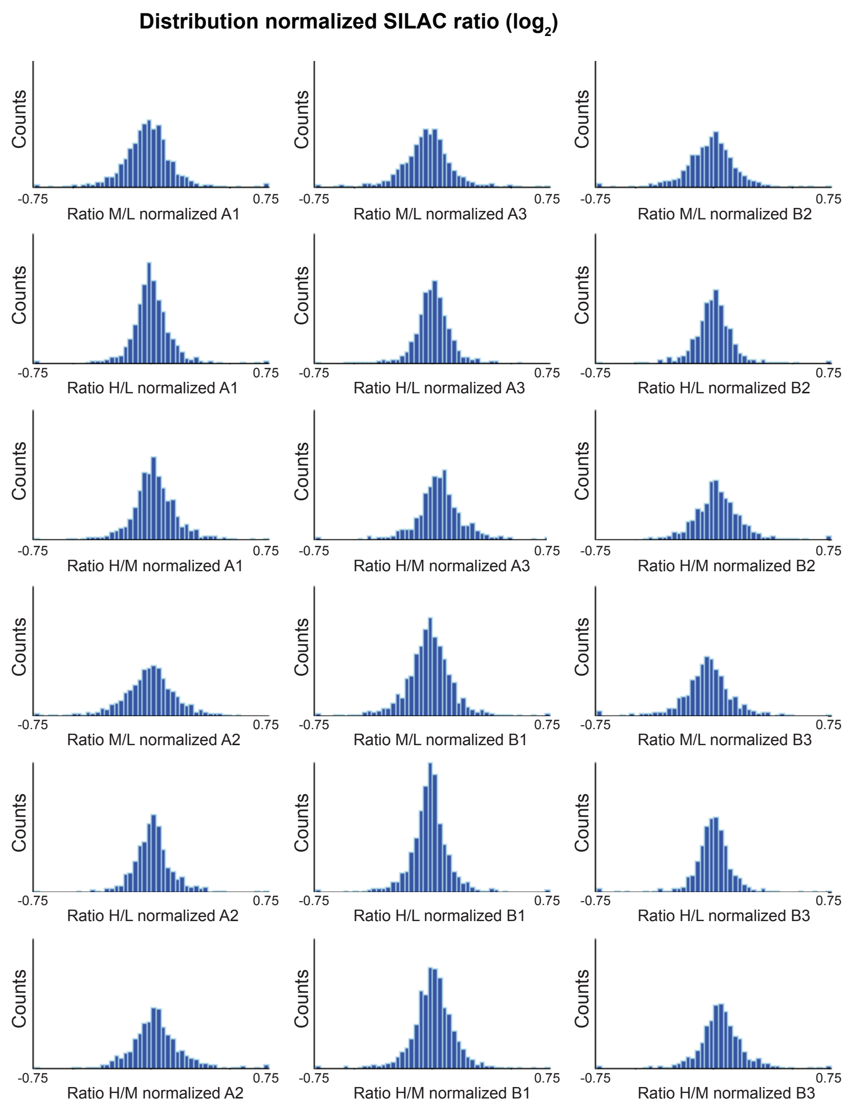


Figure S2



Average SILAC ratio: 0.0027
Average Standard Deviation: 0.1829